In vivo lymph node mapping by Cadmium Tellurium quantum dots in rats

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ABSTRACT

Background: Intraoperative lymph node mapping (LNM) is highly significant for many surgeries in patients with cancer. Many types of tracers are currently used, but the ideal method has not yet been identified. We aimed to identify a stable lymphatic drainage pathway in an animal model and compared the effects of quantum dots (QD), a new fluorescent tracer, with those of methylene blue in intraoperative LNM.

Materials and methods: Indian ink (0.2 mL) was subcutaneously injected into the plantar metatarsal regions of six Sprague–Dawley rats. After 2 wk of incubation and subsequent dissection, the potentially stained LNs were examined pathologically to identify the lymphatic drainage pathway. After applying anesthesia, 0.1 mL methylene blue (2%) and QD (1 mg/mL) were injected into the plantar metatarsal regions of six rats for intraoperative LNM. The QD group was observed with a near-infrared imaging system, and the methylene blue group was directly observed. Drainages were recorded at 5, 10, 30, 60, and 120 min and at 1 d.

Results: Two three-level drainage pathways, that is, a peripheral drainage (popliteal LNs, inguinal LNs, and axillary LNs) and a central drainage (popliteal lymph node [LN], iliac LN, and renal LN) pathways were identified. Both methylene blue and QD stained the sentinel lymph node (SLN) quickly, but methylene blue was difficult to identify in the deep tissues and the LNs beyond the SLN. Furthermore, the blue-stained LNs remain dyed for only 2 h. In contrast, the QDs exhibited high target-to-background ratios in both the SLNs and the following LNs. Additionally, the fluorescence lasted from 5 min–1 d after injection.

Conclusions: An ideal lymphatic drainage model was found. QDs are excellent tracers for intraoperative LNM compared with methylene blue. Near infrared fluorescent imaging is a promising LNM method for clinical practice.

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1. Introduction

Lymphatic metastasis is one of the main metastatic pathways of most cancers and determines the prognoses of those cancers to a large extent. The sentinel lymph node (SLN) is defined as the first lymph node (LN) to receive the lymphatic drainage from a primary malignancy and was first discovered by Morton et al. [1] in melanoma patients. Theoretically, the SLN can help to realize the status of regional LNs. Techniques for sentinel lymph node biopsy has been developed and successfully applied in breast cancer and melanoma and allow for precise staging and the collection of prognostic information with minimal morbidity [2].

Extensive lymphadenectomy increases patient morbidity and mortality, and the optimal extents of lymphadenectomy for many types of cancers remain unclear. Furthermore, the lymphatic drainage of the gastrointestinal tract is unclear due to the complicated embryonic development of the tract. Unexpected drainage pathways are often found clinically [3]. Compared with the sentinel lymph node biopsy technique, lymph node mapping (LNM) should identify LNs more readily than SLNs. The ideal LNM technique should be rapid, noninvasive, and produce lasting signal brightness. Current LNM methods include preoperative lymphoscintigraphy with technetium-99 m sulfur colloid, intraoperative mapping with blue dye, and a combination of these two techniques. However, LNM with organic dyes produces poor tissue contrast that is difficult to detect in deep anatomic regions, such as the retroperitoneal space. Radioactive tracers require a longer time to migrate to the LN, which makes them inappropriate for LNM. Furthermore, high radioactivity at the injection site can interfere with detection [4].

Recently, near infrared (NIR) fluorescence has received significant attention for its superior penetration of the surrounding tissue and lower autofluorescence contamination [5]. Quantum dots (QDs) are an emerging fluorescent material. These novel semiconductors possess the following unique electronic and optical properties: size-tunable light emission, superior signal brightness, resistance to photo-bleaching, and broad absorption spectra [6]. QDs have been widely used in bio-imaging both in vitro and in vivo [7–10]. However, most of the available studies have been restricted to the study of SLNs, and research regarding LNM is lacking. We aimed to identify a stable animal lymphatic drainage pathway and explore the potential use of QDs in LNM.

2. Methods

2.1. Preparation of the QDs

The method for the preparation of QDs has previously been introduced [11] and verified in a previous animal study [7]. In brief, 100 mg of sodium borohydride (NaBH₄) was dissolved in distilled water. After degassing for 0.5 h under nitrogen flow in an ice-water bath (4 °C), 127 mg (1 mmol) of black tellurium powder was rapidly added. Sodium hydrogen telluride was produced under a continuous nitrogen flow with vigorous stirring for 12 h.

Meanwhile, 100 mL of a solution of 366.6 mg (2 mmol) cadmium chloride (CdCl₂) containing 382.1 mg (3.6 mmol) 3-mercaptopropionic acid (3-MPA) with a concentration of Cd₂⁺ of 2 mmol/L and a molar ratio of Cd₂⁺ to 3-MPA of 1:1.8 was made. The pH value was adjusted to 9 through the addition of 2 mol/L of NaOH drops. Next, 1 mL of sodium hydrogen telluride was added to the 20 mL CdCl₂ solution under vigorous stirring. Finally, 8 mL of the mixture solution was kept in a Teflon-lined stainless steel autoclave at 185 °C for 30–60 min and cooled to room temperature after hydrocooling. The product was washed three times with ethanol and then placed into a vacuum drying oven at 40 °C. Then, the QDs were obtained.

2.2. Electron microscopy and spectrum analyses

The QDs were diluted in deionized water. A few drops were placed on a piece of carbon film. After volatilization, pictures were acquired under electron microscopy in the 200-V stem mode.

Diluted QDs were placed under a spectrofluorimeter with a 450 nm excitation wavelength. The curves of the spectra were drawn according to the intensity of each nanometer of emission light between 550 nm and 800 nm.

2.3. Identification of the anatomies of the possibly related LNs

Adult male Sprague–Dawley rats weighing 200 ± 20 g were used. This study was approved by the Animal Care and Use Committee of Fudan University and performed following the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No.86-23, revised 1996).

Indian ink (0.2 mL) was subcutaneously injected to the planter metatarsal regions of six rats. After incubation for 2 wk, the rats were killed by cervical dislocation and dissected to check for the presence of dyed LNs and lymphatic vessels in the drainage areas.

2.4. Intraoperative observation of lymphatic drainage

The 12 rats were anesthetized with 2.5% pentobarbital (40 mg/kg) intraperitoneally and were divided evenly and randomly into two groups under the same circumstances. The rats in group A were subcutaneously injected with 0.1 mL methylene blue (2%) into the plantar metatarsal of the right posterior limb. The statuses of the related LNs were recorded at 5, 10, 30, 60, and 120 min and 1 d.

With the same method described previously, the same volume of QDs (1 mg/mL) was used for group B. The images were acquired with an NIR imaging system (NightOwl ILI893 NC320; Berthold Technologies GmbH & Co KG, Bad Wildbad, Germany) with an excitation wavelength of 450 nm and an emission wavelength of 700 nm.
2.5 Pathologic identification of resected tissue

All stained or shining tissues observed in the imaging system were resected after observation for pathologic identification. After embedding with Tissue-Tek O.C.T. compound (Sakura Finetek USA, Inc, Torrance, CA) and frozen in liquid nitrogen, the LNs were sent for hematoxylin-eosin staining.

3. Results

3.1 Physical characteristics of QD

The average size of the QDs was 3.5 ± 0.30 nm, according to the transmission electron microscopy results shown in Figure 1A. The emitting wavelength was between 580 and 800 nm, and the peak was at approximately 680 nm as shown in Figure 1B.

3.2 Lymphatic drainage of the left hind footpad

The injections of India ink into the left hind footpads labeled two drainage pathways; that is, a peripheral and a central pathway. Information about the related LNs is shown in Table and Figure 2.

The peripheral drainage system contained three levels of drainage, and the involved LNs were the popliteal, inguinal, and axillary LNs. The lymphatic fluid was finally exported to the subclavian. In the central drainage pathway, at least three levels of drainage were observed because of the inconsistent presence of a para-aortic LN. The related LNs were the popliteal, iliac, para-aortic, and renal LNs. The renal duct received lymphatic fluid from the renal LN and exported it to the cisterna chyli.

3.3 Real-time mapping of the LN via intraoperative injections

For the methylene blue group, <5 min after injection, the popliteal LN was stained, and the blue staining was sustained for 2 h. After approximately 2 h, the LNs were difficult to find by color. The optimal time for observation was 1 h after injection. Furthermore, the second-level LNs were minimally stained after injection. Figures 3 and 4 show the LNs 1 h after injection. Almost none of the inguinal LNs were stained during the observation, which demonstrated that the central pathway was the main drainage system.

In the QD group, the intrinsic autofluorescence of the rat tissue was minimal compared with the QD signal. The popliteal LN was visible under NIR imaging approximately 5 min after injection, and the fluorescence was sustained for at least 1 d. As shown in Figure 5, the iliac LN was visible under near infrared fluorescent (NIRF) imaging approximately 60 min after injection, and the fluorescence was sustained for at least 1 d. Moreover, the inguinal LNs and renal LNs were not visible under the NIRF system.

All the resected tissues were confirmed by hematoxylin-eosin staining as shown in Figure 6.

4. Discussion

Accurate and convenient LNM is crucial for enabling surgeons to precisely conduct minimally invasive operations [12]. In this animal model, we first identified a lymphatic drainage pathway to perform LNM in rats. The footpad is rich in capillary lymph ducts, making it an advantageous site for the study of lymphatic drainage. In our study, steady lymphatic drainage was identified and checked in our LNM experiment. The injection of Indian ink into the left hind footpad identified

### Table – The anatomy and the efferent drainage of related LNs.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Number</th>
<th>Location</th>
<th>Efferent drainage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Popliteal LN</td>
<td>1</td>
<td>Popliteal fossa</td>
<td>Inguinal LNs and iliac LN</td>
</tr>
<tr>
<td>Inguinal LN</td>
<td>2</td>
<td>In the flank</td>
<td>Axillary LNs</td>
</tr>
<tr>
<td>Axillary LN</td>
<td>2</td>
<td>The axilla (medial to the shoulder and dorsolateral to the pectoralis)</td>
<td>Subclavian duct</td>
</tr>
<tr>
<td>Iliac LN</td>
<td>1</td>
<td>The junction between abdominal aorta and the common iliac artery</td>
<td>Renal LNs or para-aortic LN</td>
</tr>
<tr>
<td>Para-aortic LN*</td>
<td>0 or 1</td>
<td>Adjacent with the abdominal aorta</td>
<td>Renal LNs</td>
</tr>
<tr>
<td>Renal LN</td>
<td>1</td>
<td>Dorsal to the ipsilateral renal veins</td>
<td>Cistern chyli</td>
</tr>
</tbody>
</table>

*1/6 has 2 para-aortic lying laterally to the abdominal aorta, 1/6 has only one para-aortic LN and, the remaining 4/6 rats have no para-aortic LNs.
Fig. 2 – The anatomy of the related LNs. (A–C) show the peripheral system and (D) shows the central system. a: popliteal LN, b: inguinal LNs, c: axillary LNs, d: iliac LN (stained), e: para-aortic LN (stained), f: renal LN (stained), g: iliac LN (unstained), and h: para-aortic LN (unstained). (Color version of figure is available online.)

Fig. 3 – In vivo LNM with methylene blue. (A) shows the LNM in popliteal fossa and (B,C) show the LNM in abdominal cavity. a: popliteal LN, b: iliac LN (stained), c: iliac LN (unstained), and d: renal LN (stained). (Color version of figure is available online.)
two drainage pathways; that is, a peripheral and a central pathway. In our study, the inguinal LNs were not stained by methylene blue even after dilution, and a similar result was observed in the QD group. For this reason, the central drainage pathway might be the main route. As expected, for LNM in sites with complicated lymphatic drainage situations, it was necessary image the main drainage pathway.

The ideal LNM technique should be rapid, noninvasive, nonradioactive, and produce sustained signal brightness. In our study, the QDs and methylene blue exhibited similar results during the observation period. However, the QDs were better tracers than was the methylene blue. First, the QDs exhibited a better signal to background ratio, particularly in the secondary LNs shown in Figures 3 and 5. It was difficult for the operators to distinguish the iliac LNs from the surrounding adipose tissue in the methylene blue group. Second, the QD signal was present in high quantities even after 1 d, whereas the methylene blue signal lasted only 2 h and then became blurry. The concentration of methylene blue used in our study was twice the amount that is routinely used in clinical practice, but it was still difficult to clearly distinguish the secondary LNs from the surrounding tissues. Third, the unexpected leakage of the QDs might leave a clear surgical field, while the methylene blue left a blurry field.

Based on the results of our study, the use of NIRF can aid the locating of LNs and improve cancer surgeries in the future, and this supposition is consistent with the findings of a clinical sentinel lymph node mapping (SLNM) study that used Indocyanine Green [13]. NIRF is a promising area of real-time imaging due to the low absorption and autofluorescence within the NIR range in tissues. NIRF can maximize penetration and minimize background interference [14]. QDs are a new fluorescent material and have been widely used in NIRF. Consistent with their inorganic metal cores and shells and surrounding hydrophilic organic coatings, QDs are aqueously soluble. Parungo et al. [15] showed the sensitivity of SLNM of the pleural space in rats using QDs. With the help of NIRF albumin and NIRF QD, real-time identification of SLNs was achieved by Knapp et al. [10] in an animal modal of invasive urinary bladder cancer. All the above findings are limited to the SLNMs. Customized to the appropriate diameters, QDs can also be used to for LNM. The optimal diameter for SLNM is between 10 nm and 100 nm [16]. Tracers with diameters between 15 and 50 nm cannot travel beyond the SLNs, and bigger tracers enter the lymphatic system with difficulty [17].

Fig. 4 — The resected LNs (b–d) and adipose tissue (a) 1 h after the injection of methylene blue. b–d indicate the inguinal LN, iliac LN, and popliteal LN, respectively. (Color version of figure is available online.)

Fig. 5 — In vivo fluorescence spectroscopy of the abdominal (A) and resected LNs (B) with the NIRF system 90 min after injection. In (B), the LNs in the right row show the following: a: iliac LN, b: renal LN, c: popliteal LN, and d: inguinal LN. (Color version of figure is available online.)
average size of the QDs in our study was 3.5 ± 0.30 nm, and this size had good dynamics in the lymphatic system.

In contrast to the nontoxicity of methylene blue, the potential limit and the promising clinical use of QDs are their toxicity. The toxicity of QDs is controversial. A recent study demonstrated the biocompatibility of anti-HER2ab-QDs for breast cancer imaging and the toxicity of nonconjugated QDs, which indicates that antibodies might control the adverse effects of QDs [18]. However, a previous study found no obvious toxicity of the QDs used in our study [7]. Furthermore, fluorescent LNs are resected in LNM during the operation. Additionally, a new type of cadmium-free fluorescent nanoparticle has been created [19]. After solving this problem, research regarding clinical applications will be designed to bridge the large gap between animals and humans.

Due to the invisible light emitted by the Cadmium Tellurium QD, we were able to locate fluorescent LNs conveniently and sensitively even after 1 d. This method has great potential practical value in intraoperative LNM for cancer patients. Because of the long signal duration, the QDs could also be injected preoperatively. With the invention of new QDs that have low toxicity and new surgical instruments, this novel LNM technique will make LN resection reliable and convenient.

5. Conclusions

We found an ideal lymphatic drainage model. QDs are ideal tracers for intraoperative LNM relative to methylene blue. NIRF imaging is an LNM method with promise for clinical practice.

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Authors’ contributions: C.S. wrote the article and performed most of the animal experiments. Y.Z. performed the electron microscopy and spectrum analysis. Z.R. and W.Y. synthesized the Cadmium Tellurium quantum dots. X.L. and C.S. performed the animal experiments and the observation with the near infrared fluorescent imaging system. P.S. conceived the idea for this study. All the authors have read the article and approved the final article.

Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article. The authors report no conflicts of interest and take responsibility for the entire article.

References


Fig. 6 – Hematoxylin-eosin staining of the resected LNs (×20 magnification). The arrow shows a germinal center of an LN. (Color version of figure is available online.)


